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The Human Metallothionein Gene Family: Structure and Expression

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Metallothioneins (MTs) are encoded by a multigene family in man. We have isolated those genes and analyzed the structure of some of them. The MT-II variant is encoded by a single functional gene: MT-II_A. The MT-II_B gene is a processed pseudogene derived from a reverse transcript of MT-II mRNA. On the other hand, the MT-I class of variants are encoded by a large number of genes, arranged in tandem. The MT-II_A and the MT-I_A genes show a differential response to glucocorticoid hormones and heavy metals, yet they are both expressed in primary human fibroblasts and in HeLa cells. Expression of both of those genes, in high level after transfer on bovine papilloma virus vectors, leads to increased resistance of the host cells to cadmium-induced toxicity.

Introduction

Metallothioneins (MTs) are a group of low molecular weight heavy metal binding proteins, unique in their high cysteine content. MTs specifically bind heavy metals such as zinc, cadmium, copper and mercury (*1*). MTs were discovered in 1957 by Margoshes and Valee (*2*), who have isolated a Cd^{2+} and Zn^{2+} binding protein from horse kidney. In 1966 Pulido et al. (*3*) isolated MTs from human liver. Since then, MTs were isolated and characterized from a larger number of species.

In 1964, Piscator (*4*) found that MT was present in elevated levels in the liver of rabbits exposed to cadmium. He postulated that the biosynthesis of the protein is induced by Cd^{2+} and that MT acts as a detoxifying agent by binding of Cd^{2+}. The induction of MTs after administration of various heavy metal ions was demonstrated in many different animal species and in cultured cells (*1*). In 1979 Karin and Herschman (*5*) demonstrated that the synthesis of MTs in cultured cells is induced not only by heavy metal ions, but also by glucocorticoid hormones. This finding strongly supports the proposal that MTs have an important role in regulation of trace element metabolism and, that glucocorticoids by elevating the rate of synthesis of MTs, control Cd^{2+} and Zn^{2+} homeostasis.

In this paper, we will review our studies concerning the structure and expression of the genes encoding MTs in man.

Structure of the Human MT Gene Family

To define the structure and understand the regulation of human MT genes, it was necessary to obtain cDNA clones of human MT mRNA. A good source of human MT mRNA are HeLa cells which were found to express high levels of MTs in response to Cd^{2+} (*6*). We have constructed a cDNA library from Cd^{2+} induced HeLa cells and by differential hybridization isolated a number of cDNA clones which preferentially hybridized to mRNA from Cd^{2+} induced cells. Using a mouse MT-I cDNA clone (*7*), we have identified among those clones several cDNA clones of human MT mRNA. The full nucleotide sequence of one of the clones was determined and it was found to be a nearly full-length cDNA clone of human MT-II mRNA (*8*). The coding region of that cDNA was used as a hybridization probe for detection of human MT genes in digests of human genomic DNA and their isolation from a genomic DNA library. To our surprise, we found human DNA to contain at least 12 different EcoRI fragments

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hybridizing to the coding region probe. Also the frequency of bacteriophage clones hybridizing to the same probe was about 11 per genome equivalent, suggesting that in man MTs are encoded by a multigene family (*9*). To identify those genes encoding the MT-II form (*10*) we have screened all of the genomic clones isolated, for hybridization to the 5′ and 3′ untranslated regions of MT-IIcDNA. Only three of the genomic clones were positive by these criteria: one of them contained the functional MT-II_A gene and two of them were overlapping clones containing a processed pseudogene named MT-II_B (*9*). We are fairly confident that the MT-II_A gene is the only functional human MT-II gene. Complete nucleotide sequence determination has demonstrated the presence of an open reading frame, interrupted by two introns, encoding the MT-II form (*9*).

The MT-II_B is a nonfunctional processed gene derived from MT-II mRNA, by its conversion into a full length cDNA by a yet unidentified enzymatic activity in the germ line, and the integration of that cDNA into a staggered break in the genomic DNA. Although the MT-II_B gene encodes a potentially functional MT, it is not expressed (*9*), due to the absence of a promoter element (M. Karin, unpublished).

More recently we have begun the characterization of genomic clones containing MT-I like genes. The MT-I genes are tightly clustered and, so far, we have identified seven such genes, tandemly iterated within a 30 Kb region. Not all of those genes are necessarily functional and, so far, we have found two of them to be pseudogenes due to the presence of frameshift and nonsense mutations resulting in the introduction of premature termination codons (R. Richards and M. Karin, unpublished). However, one of the genes, named MT-I_A was found to be a functional gene by using an assay described in the next section.

Increased Resistance to Cd^{2+} of Cells Expressing High Levels of MT

Bovine papilloma virus (BPV) is a DNA tumor virus with several unique properties making it useful as a mammalian plasmid vector. First, this virus does not integrate into the cellular genome and instead it maintains itself as a free replicating nuclear plasmid. Second, it does not lead to the lysis of the cells in which it replicates (*11*). We have used these properties of the virus and ligated a fragment of it to the cloned human MT-II_A gene to generate the vector pMTII-BPV. Cells transformed with this vector express high levels of human MT-II mRNA and protein. That expression is regulated by the concentration of Cd^{2+} in the growth medium, even though the human gene is present in an episomal state. Moreover, due to the high level of expression of human MT-II, the transformed cells become highly resistant to Cd^{2+}. In fact, while the relative plating efficiency of the parental cells drops to 10% at 1 μ M Cd^{2+}, the relative plating efficiency of cells containing pMTII-BPV drops to 10% only at 25 μ M Cd^{2+} (*12*). Actually, the cells transformed with pMTII-BPV are Zn^{2+} dependent due to the high level of synthesis of human MT-II (about 12% of the soluble protein).

In the absense of any recognized enzymatic activity, we consider the increased Cd^{2+} resistance of cells harboring BPV-MT plasmids, a criteria for the functionality of a MT gene and the protein it encodes. This assay has turned out to be quite useful in the characterization of human MT genes. For example, we have recently determined the full nucleotide sequence of the MT-I_A gene (R. Richards and M. Karin, in preparation) and found the amino acid sequence of the encoded protein to deviate from the published sequence of human MT-I's (*13*). We were concerned whether this gene is functional or not and therefore have inserted it into the BPV vector. Mouse cells transformed with this vector (pMTI_A-BPV) were found to be Cd^{2+}-resistant, indicating that the MT-I_A gene is functional (R. Richards and M. Karin, in preparation). This illustrates the usefulness of the BPV vector system to the functional analysis of a multigene family. Since the MT-BPV transformed cells produce very high levels of MT, they can serve as a convenient source for the isolation of large amounts of relatively homogenous MT variants for biochemical studies.

Expression of Human MT Genes

Addition of Cd^{2+}, Zn^{2+} and Cu^{2+} to the culture medium of human HeLa or primary fibroblast cells leads to rapid induction of MT mRNA (*14* and Richards and Karin, in preparation). The same mRNA's species are also induced by the addition of glucocorticoid hormones (*14* and M. Karin, unpublished). Both heavy metal ions and hormonal inductions are considered to be primary induction responses, since they lead to an increase in mRNA levels even in the absence of concurrent protein synthesis (*15*). Palmiter and co-workers have demonstrated that in mouse cells both classes of inducers act by increasing the transcription rate of the MT-I gene (*16,17*). Recently, we found that the human MT-I_A and MT-

II_A genes respond differently to different inducers, indicating possible functional differences between those genes (R. Richards and M. Karin, in preparation).

The kinetics of induction of MT mRNA by a typical heavy metal ion inducer such as Zn^{2+} and dexamethasone, a synthetic glucocorticoid, are different. Both inducers lead to rapid induction of MT mRNA, but while the response to metal is biphasic and the level of the mRNA declines after prolonged exposure, the response to the hormone is persistent and once a new elevated steady state level of MT mRNA is achieved, it is maintained as long as the hormone is present (*14,18*). The transient induction observed with Zn^{2+} is not due to a cytotoxic effect of the metal or to disappearance of the inducer due to MT induction. Another difference between the two responses is in the way they are affected by inhibition of protein synthesis. While hormonal induction of MT mRNA is only slightly elevated in the absence of protein synthesis, the induction by Zn^{2+} is augmented about threefold. Also, the basal level of MT mRNA is increased threefold by inhibition of protein synthesis (*15*). Mayo and Palmiter found that the cycloheximide effect is exerted at the level of transcription, by comparing transcription rates of the mouse MT-I gene in the presence and absence of cycloheximide (*19*).

These observations led us to suggest that (a) heavy metal ions and glucocorticoid hormones increase the transcription rates of MT genes by different mechanisms and (b) heavy metal induction is mediated through the removal of a repressor of MT gene expression. However, as a consequence of metal induction the level of the repressor is increased causing a transient induction response. Inhibition of the synthesis of this putative repressor by cycloheximide causes a large augmentation of the induction response to metal ions. In our simplest working hypothesis the repressor is apothionein (see Fig. 1), which explains the specificity of the induction response, since only metal ions that bind to the apoprotein can lead to induction. This induction is due to the conversion of apotheionein, the active repressor form, to metallothionein, the inactive repressor form. After a while, the level of MT in the cell is increased, but so does the level of the apoprotein, the putative repressor, leading to a decrease in the transcription rate of the MT genes, and resulting in a transient induction response. Induction by glucocorticoids bypasses this pathway and leads to persistent activation of the genes, as long as, the hormone receptor complex is bound to DNA (*18*).

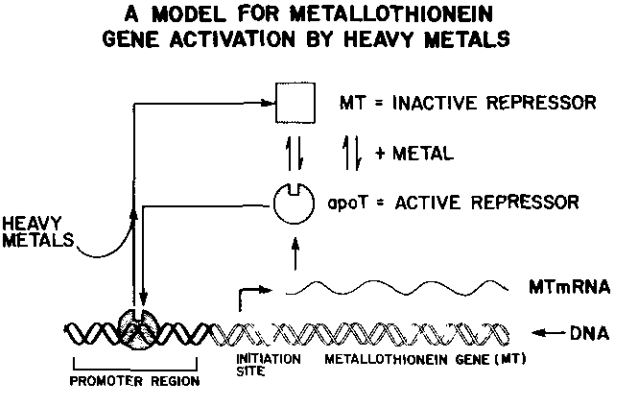

FIGURE 1. A model for metallothionein gene activation by heavy metals.

More recently we have compared the expression of the MT-II_A and the MT-I_A genes in human cells. We find that while the MT-II_A gene is induced to the same extent by Cd^{2+}, Zn^{2+}, Cu^{2+} and dexamethasone, the MT-I_A gene is only fully induced by Cd^{2+}. These results suggest that the various MT genes are regulated differently from each, most likely due to the different functions they serve.

Localization of the Regulatory Elements of Human MT Genes

As indicated, both the human MT-I_A and the MT-II_A genes are still inducible by heavy metal ions after transfer into rodent cells on BPV derived sectors (*12*). While being appropriate for studying metal induction, this sytem is not suitable for studying the hormonal regulation of cloned MT genes, since the episomal state precludes glucocorticoid induction. On the other hand, cotransfer (with the Herpes simplex virus thymidine kinase gene) of the human MT-II_A gene, into rat fibroblasts leading to stable integration into the host genome, results in retention of full responsiveness to both glucocorticoid hormones and heavy metal ions.

To test whether the regulatory DNA elements controlling MT-II_A gene expression are found within the 800 bp 5′ flanking region of the cloned fragment used in the transfection experiments mentioned above, we have fused this region to the structural portion of the Herpes simplex virus (HSV) thymidine kinase (TK) gene. The transcription of TK mRNA from this fusion gene (hMTK) is under the control of the human MT-II_A gene promoter. The hMTK fusion gene was introduced into TK^- rat fibroblasts by transfection and

selection for the TK^+ phenotype. The expression of TK mRNA in those cells was found to be both heavy metal and hormone inducible. These results indicate that the important regulatory elements for both heavy metal and glucocorticoid inductions are found within 800 bp of the 5′ flanking region of the human MT-II_A gene. Measuring the rate of synthesis of MTK mRNA under different induction conditions demonstrated transcriptional control by both Cd^{2+} and dexamethasone (*20*). More recently by performing sequential deletions of 5′ flanking nucleotides of the hMTK gene we found that only 300 bp are sufficient for maintenance of both hormone and metal induction (M. Karin et al., unpublished).

Conclusions

By applying recombinant DNA technology to study of structure and function of MT genes in man we were able to demonstrate that: (a) this group of proteins is encoded by a large gene family, (b) different MT genes vary in their response to various inducers, (c) overexpression of MT genes leads to protection of cultured cells against the toxic effect of Cd^{2+} and (d) the regulatory DNA elements controlling the expression of the human MT-II_A gene are located within 300 bp of 5′ flanking DNA whose primary structure is known.

The differences in the response of the MT-II_A and MT-I_A gene to various inducers suggest that the two genes have different functions. Since the MT-II_A gene is the one which is highly inducible by glucocorticoids and zinc, we believe this gene serves a homeostatic role. The modulation of MT-II expression by zinc, copper and glucocorticoids help to maintain zinc and copper homeostasis. During periods of high availability, zinc and copper stimulate the transcription of the MT-II_A gene, leading to increased production of MT-II, mostly in the liver, which results in the storage of the excess amounts of those essential trace metals. Glucocorticoids, by increasing the expression of MT-II, cause mobilization of zinc and probably copper from the circulation into the liver and other organs, leading eventually to hypozincemia.

On the other hand, the expression of the MT-I_A gene, and possibly of the other MT–I genes, as well, is not highly stimulated by glucocorticoids and zinc or copper. Only cadmium seems to be a potent inducer of those genes. Therefore, we believe the MT–I genes serve a protective role. This hypothesis is supported by the multiplicity of MT–I genes in most mammals except rodents. Primates have at least four functional MT–I genes (J. Kagi and M. Kimura, personal communications). If all of those genes are activated by Cd^{2+}, this would constitute an efficient protective response, due to a massive increase in the synthesis MT–I resulting in the chelating of the toxic Cd^{2+} ions.

In the future we plan to further characterize all the different MT genes and study their organization within the genome. We hope to be able to understand the genetic mechanism which has led to the generation of multiple MT–I genes in man. We also plan to investigate whether there are any tissue-specific variations in the expression of the different human MT genes. Finally we hope to be able to reconstitute an *in vitro* induction system, consisting of cloned MT genes, a few regulatory proteins and RNA polymerase-II. By using such a system it should be possible to determine how heavy metals control gene expression.

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